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STUDIES ON STEROIDS

CCXXXIII*. SEPARATION AND CHARACTERIZATION OF BILE ACID DISULPHATES IN HUMAN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The separation of disulphates of cholate, chenodeoxycholate, deoxycholate and ursodeoxycholate and their glyco and tauro conjugates has been carried out by high-performance liquid chromatography (HPLC) on a reversed-phase column. The chromatographic behaviour of bile acid disulphates was dependent on the position of the sulphate and hydroxy groups and the structure of the side-chain. The method has been applied to the separation and characterization of disulphates in biological fluids without prior deconjugation. The disulphate fraction was obtained from a urine specimen by passing it through a Sep-Pak C_{18} cartridge, followed by group separation by ion-exchange chromatography on a lipophilic gel, piperidinohydroxypropyl-Sephadex LH-20. Subsequent resolution into individual disulphates was attained by HPLC on Radial-Pak A and Cosmosil 5C18 columns. Taurochenodeoxycholate 3,7-disulphate and taurodeoxycholate 3,12-disulphate in human urine were unequivocally identified on the basis of their behavior in HPLC using mobile phases of different pH.

INTRODUCTION

In recent years, considerable attention has been focused on the metabolic significance of the sulphation of bile acids in hepatobiliary diseases. Bile acids are usually conjugated with sulphuric acid at C-3 and excreted into urine. Recently, the existence of bile acid 7-sulphate in urine has been demonstrated in patients with primary biliary cirrhosis and congenital biliary atresia [1]. Accordingly, it seemed likely that bile acid disulphates would be potential metabolites in living animals.

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The methods commonly used for the determination of bile acid sulphates in biological fluids involve prior solvolysis and/or hydrolysis, followed by the chromatographic separation of deconjugated bile acids. These procedures, however, have inevitable disadvantages, such as the lack of reliability and the loss of information on the conjugated form. As bile acid disulphates are very polar and lacking in volatility and thermal stability, high-performance liquid chromatography (HPLC) on a reversed-phase column appears to be more suitable for the separation and determination of the disulphates. In a previous study we synthesized unconjugated and conjugated bile acid disulphates as authentic specimens by unequivocal routes [2]. This paper describes the separation of disulphates of unconjugated, glyco- and tauro-conjugated bile acids by HPLC. The method has been applied to the separation and characterization of the disulphates in urine from a patient with obstructive jaundice.

EXPERIMENTAL

High-performance liquid chromatography

The apparatus used consisted of a 6000A solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) equipped with a Uvidex 100-II ultraviolet (UV) detector (205 nm) (Japan Spectroscopic, Tokyo, Japan) and a 650-10LC fluorescence spectrophotometer (excitation wavelength 370 nm; emission wavelength 470 nm) (Hitachi, Tokyo, Japan). The test samples were applied to the chromatograph by a U6K sample loop injector (Waters Assoc.) with an effective volume of 2 ml. Cosmosil 5C18 (5 μm; 15 cm$\times$4.6 mm I.D.) (Nakarai Kagaku, Kyoto, Japan) and Radial-Pak A (10 μm; 10 cm$\times$8 mm I.D.) (Waters Assoc.) columns were used at ambient temperature.

Materials

Bile acid 3,7-, 3,12- and 7,12-disulphates were synthesized in these laboratories by previously reported methods [2]. Sephadex LH-20 was purchased from Pharmacia (Uppsala, Sweden). All chemicals employed were of analytical-reagent grade. Solvents were purified by distillation prior to use. Piperidinohydroxypropyl-Sephadex LH-20 (PHP-LH-20) (acetate form, 0.6 mequiv./g) [3] and 1-anthroyl nitrile [4] were prepared in the manner previously reported. A Sep-Pak C_{18} cartridge (Waters Assoc.) was washed successively with ethanol (10 ml) and water (10 ml) prior to use. All glassware was silanized with trimethylchlorosilane.

Separation and characterization of bile acid disulphates in human urine

A urine sample (4 ml) from a patient with obstructive jaundice was diluted with 0.5 *M* sodium phosphate buffer (pH 7.0, 5 ml) and passed through a Sep-Pak C_{18} cartridge. After washing with water (10 ml), bile acids were eluted with 90% ethanol (4 ml). The eluate was applied to a column (18 mm$\times$6 mm I.D.) of PHP-LH-20 (100 mg). Elution was carried out at a flow-rate of 0.2 ml/min. After removal of neutral compounds by washing with 90% ethanol (4 ml), bile acids were separated into unconjugated, glyco- and tauro-conjugated and monosulphate fractions by stepwise elution with 0.1 *M* acetic acid in 90% ethanol (4

ml), 0.2 *M* formic acid in 90% ethanol (4 ml), 0.3 *M* acetic acid–potassium acetate in 90% ethanol (pH 6.3) (4 ml) and 0.2 *M* acetic acid–ammonium acetate in 90% ethanol (pH 8.5) (6 ml). The desired bile acid disulphates were then eluted with 0.3 *M* acetic acid–ammonium acetate in 90% ethanol (pH 9.5) (4 ml) and the eluate was evaporated in vacuo below 40°C. The residue obtained was applied to a Sep-Pak C_{18} cartridge in the manner described above for removal of inorganic salts. Further purification was performed by HPLC on Radial-Pak A using 0.3% ammonium phosphate buffer (pH 7.0)–methanol (3:2) as the mobile phase. The eluate corresponding to the disulphates on the chromatogram was collected and subjected to HPLC on Cosmosil 5C18. Bile acid disulphates were monitored by a UV detector at 205 nm.

Characterization of bile acids in hydrolysate

The eluate corresponding to each peak on the chromatogram was subjected to solvolysis with dimethoxypropane–water–concentrated hydrochloric acid according to the procedure previously reported [5]. Unsulphated bile acids thus obtained were treated with 1-anthroyl nitrile in the presence of quinuclidine in acetonitrile at 60°C for 20 min [6]. The 3-(1-anthroyl) derivatives were separated on PHP-LH-20 into unconjugated, glyco- and tauro-conjugated fractions. Each fraction was then subjected to HPLC on a Cosmosil 5C18 column using 0.3% potassium phosphate buffer–methanol as the mobile phase. The eluate was monitored by fluorescence detection (excitation wavelength 370 nm; emission wavelength 470 nm).

RESULTS AND DISCUSSION

Chromatographic behaviour of bile acid disulphates

Initially, the effect of the pH of the mobile phase on the capacity ratio (k') was investigated on the Cosmosil 5C18 column with a 0.3% ammonium phosphate buffer–methanol system. The k' values of the disulphates of cholate, chenodeoxycholate, deoxycholate and ursodeoxycholate relative to taurochenodeoxycholate 3,7-disulphate were plotted against pH (Fig. 1). The relative k' values were influenced by the pH of the mobile phase. In the pH range 6.5–7.5, unconjugated, glyco- and tauro-conjugated bile acid disulphates exhibited similar k' values. On the other hand, the k' values of unconjugated and glyco-conjugated disulphates increased with decreasing pH from 6.0 and 4.5, respectively. A similar phenomenon has previously been observed with bile acids, their monosulphates and 3-glucuronides [7–9], and such chromatographic behaviour can be explained in terms of the dissociation of the acidic group at C-24. Irrespective of the type of conjugation, disulphates were eluted earlier with increasing number of hydroxy group on the steroid nucleus. Unconjugated, glyco- and tauro-conjugated ursodeoxycholates exhibited much smaller k' values than corresponding dihydroxylated bile acids and nearly identical k' values with corresponding cholates. This is probably due to the equatorial nature of the 7β-hydroxy group which exerts a decrease in hydrophobic binding between β-face of the steroid nucleus and the stationary phase [10].

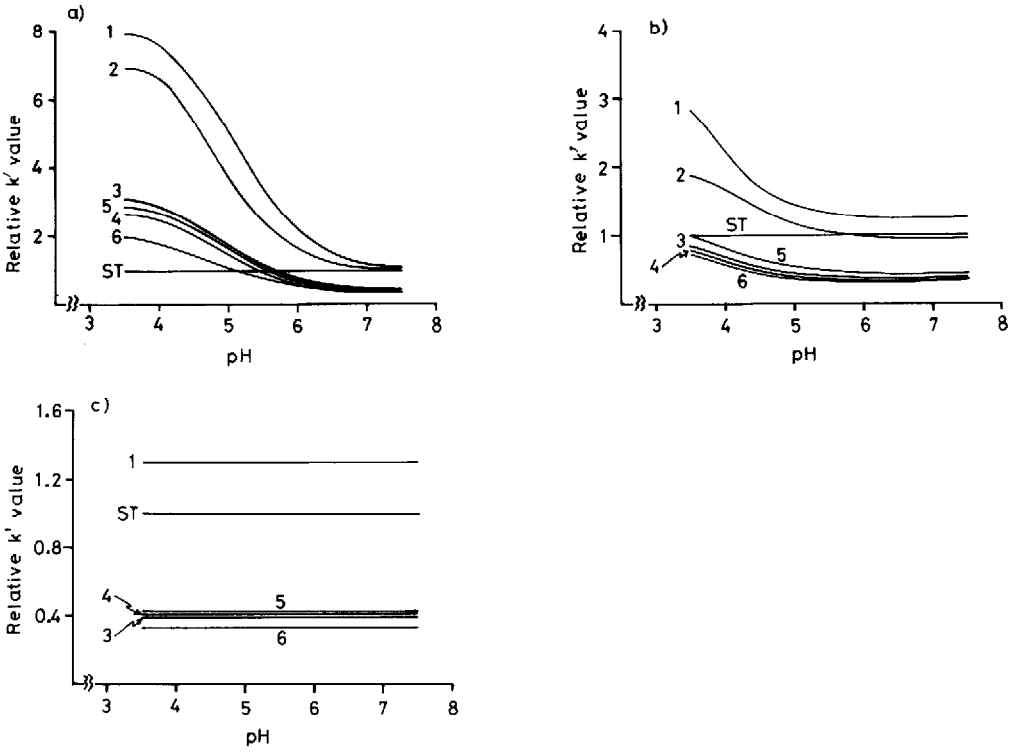

Fig. 1. Effect of pH of mobile phase on k' values of bile acid disulphates relative to taurochenodeoxycholate disulphate. 1 = Deoxycholate 3,12-disulphate; 2 = chenodeoxycholate 3,7-disulphate; 3 = ursodeoxycholate 3,7-disulphate; 4 = cholate 3,7-disulphate; 5 = cholate 3,12-disulphate; 6 = cholate 7,12-disulphate; ST (standard) = taurochenodeoxycholate 3,7-disulphate. (a) Unconjugated; (b) glyco-conjugated; (c) tauro-conjugated bile acids. Column, Cosmosil 5C18; mobile phase, 0.3% ammonium phosphate buffer–methanol; flow-rate, 1.0 ml/min; detection, 205 nm.

The k' values of cholate 7,12-disulphates were smaller than those of cholate 3,7- and 3,12-disulphates. It has previously been shown that the steric interaction arises between the 12α-sulphate group and the acidic group of the side-chain at higher pH [11]. The effect of the pH of the mobile phase on the chromatographic behaviour of cholate disulphates was further investigated on Cosmosil 5C18 with a 0.3% ammonium phosphate buffer–acetonitrile system. The k' values of unconjugated, glyco- and tauro-conjugated cholate 3,12- and 7,12-disulphates relative to the corresponding cholate 3,7-disulphate were plotted against pH (Fig. 2). The relative k' values of unconjugated cholate 3,12- and 7,12-disulphates decreased with decreasing pH. On the other hand, the pH effect was not observed with glyco- and taurocholates in this pH region. This result strongly implies the presence of steric interaction between the 12α-sulphate group and the acidic moiety of the side-chain at C-24 at higher pH.

The effect of salt concentration on the resolution of glyco-conjugated chenodeoxycholate and deoxycholate disulphates was examined using ammonium phosphate buffer (pH 7.0)–methanol as the mobile phase. The resolution (R_s)

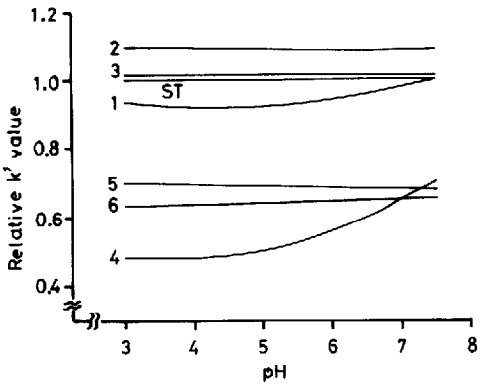

Fig. 2. Effect of pH of mobile phase on k' values of cholate 3,12- and 7,12-disulphates relative to cholate 3,7-disulphates. 1 = Cholate 3,12-disulphate; 2 = glycocholate 3,12-disulphate; 3 = taurocholate 3,12-disulphate; 4 = cholate 7,12-disulphate; 5 = glycocholate 7,12-disulphate; 6 = taurocholate 7,12-disulphate; ST = unconjugated, glyco- and tauro-conjugated cholate 3,7-disulphates. Mobile phase, 0.3% ammonium phosphate buffer–acetonitrile. Other conditions as in Fig. 1.

increased with increasing salt concentration in the mobile phase, and the maximum R_s value was ca. 1.3 at a 0.3–0.5% salt concentration, where the two peaks should be completely resolved. These data indicate that the combined use of 0.3% ammonium phosphate buffer (pH 3.5)–methanol (24:26) and 0.3% ammonium phosphate buffer (pH 7.0)–methanol (24:23) as mobile phases is preferable for the separation of bile acid disulphates.

Group separation of bile acid disulphates on PHP-LH-20

Elimination of bile acid monosulphates which exist predominantly in urine is a prerequisite for the reliable analysis of the disulphates. It has previously been demonstrated that bile acids are easily fractionated into groups according to their conjugated forms using lipophilic anion-exchange gels, diethylaminohydroxypropyl-Sephadex LH-20 [12,13] and PHP-LH-20 [14]. In this study, the use of PHP-LH-20 with acetic acid–ammonium acetate in 90% ethanol as the eluent was undertaken for the separation of mono- and disulphated bile acids. A synthetic mixture of 50 μg each of unsulphated, 3-monosulphated and disulphated bile acids dissolved in 90% ethanol was applied to a column of PHP-LH-20. After washing with 90% ethanol to remove neutral compounds, unsulphated bile acids were eluted successively with 0.1 M acetic acid in 90% ethanol, 0.2 M formic acid in 90% ethanol and 0.3 M acetic acid–potassium acetate in 90% ethanol (pH 6.3). The desired mono- and disulphate fractions were then eluted with 0.2 M acetic acid–ammonium acetate in 90% ethanol (pH 8.5) and 0.3 M acetic acid–ammonium acetate (pH 9.5), respectively. As illustrated in Fig. 3, unconjugated, glyco- and tauro-conjugated, monosulphated and disulphated bile acids were distinctly resolved into five groups.

Separation and characterization of bile acid disulphates in human urine

The separation and characterization of bile acid disulphates in urine was carried out according to the scheme shown in Fig. 4. The urine sample from a patient

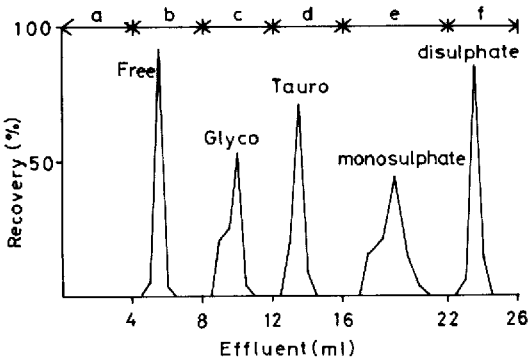

Fig. 3. Group separation of bile acids on PHP-LH-20. Eluent: (a) 90% ethanol; (b) 0.1 *M* acetic acid in 90% ethanol; (c) 0.2 *M* formic acid in 90% ethanol; (d) 0.3 *M* acetic acid–potassium acetate in 90% ethanol (pH 6.3); (e) 0.2 *M* acetic acid–ammonium acetate in 90% ethanol (pH 8.5); (f) 0.3 *M* acetic acid–ammonium acetate in 90% ethanol (pH 9.5).

with obstructive jaundice was extracted with a Sep-Pak C_{18} cartridge and then subjected to group separation on PHP-LH-20. The disulphate fraction thus obtained was further purified by HPLC on a Radial-Pak A column using 0.3% ammonium phosphate buffer (pH 7.0)–methanol (3:2) to remove co-existing substances. The eluates corresponding to k' values of 3.0–6.5 (F_1), 12.0–15.0 (F_2) and 16.0–20.0 (F_3) were collected (Fig. 5).

Previously, we investigated the chromatographic behaviour with mobile phases of various pH and found that they were dependent on the position of the sulphate and hydroxy groups and the structure of the side-chain. This finding was applied to the structural characterization of bile acid disulphates in human urine. After addition of an internal standard, the eluate was subjected to HPLC on Cosmosil 5C18 employing three mobile phases of different pH. It is evident from the data in Table I that the relative k' values of two bile acid disulphates in urine were identical with those of authentic taurochenodeoxycholate and taurodeoxycholate disulphates.

In previous work, we developed a new fluorescence labelling reagent, 1-anthroyl nitrile, and demonstrated its utility for selective derivatization of bile acids through the 3α-hydroxy group [6]. The identity of these disulphates was further confirmed by degradative means. The eluate corresponding to each peak on the chromatogram was subjected to solvolysis with dimethoxypropane–water–concentrated hydrochloric acid according to the procedure previously reported [5]. After addition of an internal standard, the bile acids liberated were treated with 1-anthroyl nitrile. The 3-(1-anthroyl) derivatives were resolved into unconjugated, glyco- and tauro-conjugated fractions on PHP-LH-20, and each fraction was subjected to HPLC on Cosmosil 5C18 with three different mobile phases. As shown in Table II, the relative k' values of disulphated bile acids were identical with those of corresponding authentic samples.

It is obvious from these results that taurochenodeoxycholate and taurodeoxycholate are present in urine as disulphates. No other bile acid disulphates could

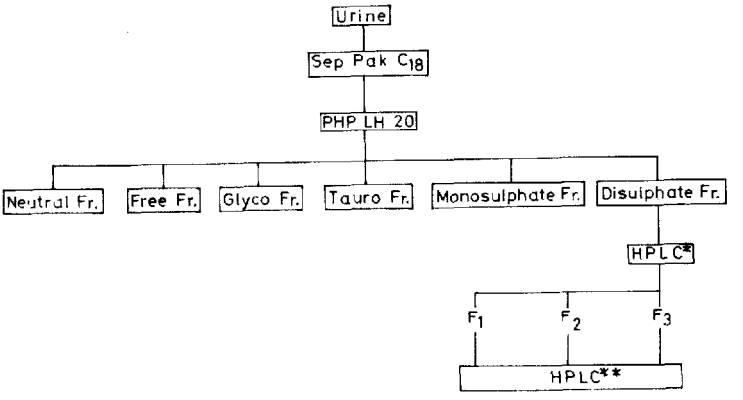

Fig. 4. General scheme for the separation and characterization of bile acid disulphates in human urine. Each fraction corresponds to the following bile acid disulphates: (F_1) cholate and ursodeoxycholate disulphates; (F_2) chenodeoxycholate disulphates; (F_3) deoxycholate disulphates. Conditions: * Radial-Pak A, 0.3% ammonium phosphate buffer (pH 7.0)-methanol (3:2); ** Cosmosil 5C18, 0.3% ammonium phosphate buffer (pH 3.5, 5.0, 7.0)-methanol (24:26, 24:24, 24:23).

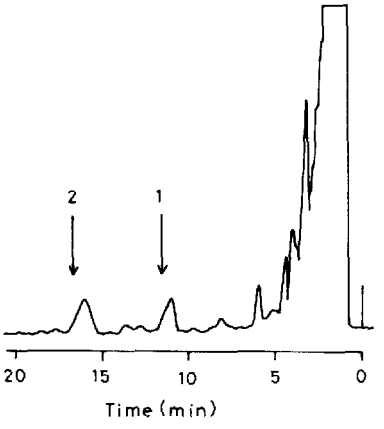

Fig. 5. Separation of bile acid disulphates in human urine by HPLC. Column, Radial-Pak A; mobile phase, 0.3% ammonium phosphate buffer (pH 7.0)-methanol (3:2). Peaks: 1 = taurochenodeoxycholate 3,7-disulphate; 2 = taurodeoxycholate 3,12-disulphate.

TABLE I

RELATIVE k' VALUES OF BILE ACID DISULPHATES IN HUMAN URINE

The figures express the k' values relative to the internal standard. Column, Cosmosil 5C18; mobile phase, 0.3% ammonium phosphate buffer–methanol; flow-rate, 1.0 ml/min.

Bile acid disulphate	Internal standard	k'		
		pH 3.5	pH 5.0	pH 7.0
Taurochenodeoxycholate 3,7-disulphate	Taurodeoxycholate 3,12-disulphate	0.76	0.77	0.76
Urine		0.76	0.77	0.76
Taurodeoxycholate 3,12-disulphate	Taurochenodeoxycholate 3,7-disulphate	1.32	1.30	1.31
Urine		1.32	1.30	1.30

TABLE II

RELATIVE k' VALUES OF HYDROLYSATES DERIVED FROM BILE ACID DISULPHATES IN HUMAN URINE

The figures express the k' values relative to the internal standard. Column, Cosmosil 5C18; mobile phase, 0.3% potassium phosphate buffer–methanol; flow-rate 1.6 ml/min.

Bile acid	Internal standard	k'		
		pH 3.0	pH 4.0	pH 6.2
Taurochenodeoxycholate	Taurodeoxycholate	0.84	0.85	0.86
Urine		0.84	0.85	0.86
Taurodeoxycholate	Taurochenodeoxycholate	1.19	1.18	1.17
Urine		1.18	1.18	1.17

be detected even when 40 ml of urine were subjected to the analysis. It is well substantiated that glyco-conjugated bile acids are predominant in the unsulphated fraction in addition to the monosulphated fraction. In a previous study, we established the existence of taurochenodeoxycholate 7-sulphate in the urine of patients with primary biliary cirrhosis and congenital biliary atresia [1]. It should be noted that 7- and 12-sulphated bile acids in human urine are mainly conjugated with taurine.

Further studies on the metabolism of sulphated bile acids in hepatobiliary diseases are being conducted and the results will be reported elsewhere.

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